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Cytotoxic T Cell Immunity to Human Cytomegalovirus Glycoprotein B

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Human cytomegalovirus (HCMV) is associated with significant morbidity and mortality following immunosuppression and in pregnancy. HCMV infection may be accompanied by acute disease but persists asymptomatically. Cytotoxic T lymphocytes (CTL) appear to be an important immune effector mechanism in maintaining the normal host-virus equilibrium. Glycoprotein B may be an important target for future subunit vaccines as it has been found to elicit both neutralising antibody and CTL responses.

We therefore studied the ability of normal asymptomatic HCMV-seropositive individuals and women throughout pregnancy to determine the presence of HCMV and gB-specific CTL responses. CTL effector cells were induced by stimulation of peripheral blood mononuclear cells (PBMC) with AD169 HCMV-infected cells and gB-specific CTL were identified using chromium labeled, vac.gB-infected cells.

In 7 HCMV-seropositive individuals, HCMV-specific CTL were identified. Three of the 7 individuals which lysed HCMV-infected cells lysed vac.gB-infected B cells. However, vac.gB-infected autologous fibroblasts, which only present MHC class I, were not killed. Using MHC class I single allele targets, no specific lytic response was observed, suggesting a MHC class II restricted CTL response. Flow cytometric analysis showed the gB-specific effector cell phenotype to be CD3+, CD4+, CD8−. In conclusion, a gB-specific CTL lytic response was identified in seropositive individuals which in most cases was MHC class II-restricted.

KEY WORDS: human cytomegalovirus, cytotoxic T cell, glycoprotein B

INTRODUCTION

Human cytomegalovirus (HCMV) is a large species-specific herpes virus (DNA 1.5×10^5 kDa) which is widely prevalent in the human population (with 60% of adult populations being seropositive [Krech, 1973]). As with all herpes viruses, primary infection with HCMV is invariably followed by lifelong persistence [Roizman, 1982]. Primary infection in the immunocompetent host may be associated with an infectious mononucleosis syndrome but is often asymptomatic. However, infection or reactivation in the immunocompromised host is associated with appreciable morbidity and mortality. It is assumed that the cellular immune response to HCMV plays a major role in controlling its reactivation, but the precise nature of this control in normal seropositive subjects is unknown.

The envelope of cytomegalovirus contains several glycoproteins, many of which are disulphide-linked [Gretch et al., 1988a]. One family of highly abundant glycoproteins has been designated gCI [Gretch et al., 1988b], or glycoprotein B (gB) since the protein is encoded by a gene which has homology to the gB of herpes simplex virus [Cranage et al., 1986]. The precise nature of the glycoprotein complex is unknown but it is probable that the disulphide-linked complexes found in HCMV envelopes are composed of at least two glycoproteins with molecular weights (MW) of between $50–58 \times 10^3$ and $93–130 \times 10^3$ [Britt, 1984; Gretch et al., 1988a]. HCMV gB is highly conserved among all herpes viruses in many aspects, including genomic location, amino acid sequence, secondary and tertiary structure, and it is thought in function [Rapp et al., 1993].

HCMV is now the commonest viral cause of congenital or intrauterine infection with an incidence ranging between 0.2% and 2% of live births [Stagno et al., 1977]. Ten percent of these infants are symptomatic at birth and preterm [Boppana et al., 1993]. They suffer with cytomegalic inclusion disease which is frequently fatal and manifests as hepatosplenomegaly, petechiae and abnormalities of the nervous system including chorioretinitis, optic atrophy, cerebral calcification, microcephaly, and deafness. Survivors are usually mentally handicapped with a poor long-term prognosis. Other associated defects have been described such as cardiovascular

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and gastrointestinal lesions. Of the 90% of infants who are asymptomatic at birth, 5–15% develp later nuerological sequelae such as mental retardation and deafness. In Britain, 200–600 infants a year may be affected in this way [Eleck and Stern, 1974]. Disease prevention, possibly by vaccination, is therefore desirable.

Whilst HCMV may be cultured from 10% to 30% of women in pregnancy [Montgomery et al., 1972] fetal infection is rare, pointing to the existence of protective immunological mechanisms. In addition, fetal damage after reactivation or recurrent maternal infection is less common, suggesting transfer of protection from the persistently infected mother. Antibody-mediated immunity may be important, as clinical episodes are generally less severe in infants born to mothers with circulating HCMV antibodies [Yeager et al., 1981]. However, unlike other intrauterine infections, such as rubella and toxoplasmosis, preexisting maternal humoral antibody may not control the spread of virus from the mother to the fetus [Farrer et al., 1986]. Cell-mediated immune responses, particularly MHC-restricted cytotoxic T lymphocytes (CTL), are thought to play a role in preventing HCMV-induced disease [Reddehase et al., 1987; Riddell et al., 1991]. It is possible that CTL are involved in the control of transendothelial and localised placental infection, thereby preventing and reducing the infection dose to the fetus. Therefore, it may be important for a vaccine to generate specific CTL as well as a neutralising antibody response.

Most neutralising antibodies against HCMV are directed against gB [Britt et al., 1990]. Furthermore, cell-mediated immunity, both proliferative/T helper responses [Curtsinger et al., 1993] and cytotoxic T cells (CTL), has been described. As gB is a major candidate glycoprotein, we examined gB-specific CTL in peripheral blood mononuclear cells (PBMC) of normal asymptomatic seropositive individuals to determine this fine specificity and persistence of the response in pregnancy.

MATERIALS AND METHODS

Virus Preparation

HCMV. AD169 strain HCMV (seed stock from American type tissue culture collection, cat no VR-5381) was propagated in MRC-5 cells (Flow Laboratories, Oxfordshire, UK). The medium was harvested when 80–90% cytopathic effect was present (12–14 days postinfection), cleared of cellular debris by centrifugation at 1,000 g for 10 min and the supernatant stored at −70°C. Stocks of HCMV contained 10^4–10^7 plaque-forming units (PFU/ml).

Generation of gB vaccinia recombinants. vac.gB was constructed as previously described [Cranage et al., 1986]. To generate vac.gB459 and vac.gB685 (containing amino acids 1–459 and 1–685, respectively) the gB gene was subcloned from the genomic Hind IIIf fragment (provided by Dr. G. Wilkinson, PHLS, UK) as a ECII fragment into the Sma I site of pUC12.

A stop codon and BamHI site was inserted into the internal EcoRI site (position 2208 of AD169gB), generating a truncation at amino acid 685 (i.e., lacking the transmembrane anchor [amino acids 712–776] and intracytoplasmic domain). The gB459 truncate was produced by inserting a synthetic oligonucleotide with a stop codon in all 3 ORFs: the BamHI-AvaII gB restriction fragment of the gB685 truncate was isolated and a 28bp sequence ligated: GACCAGAAGATAACTGATAAG-GATCCGGTCTTCTATTGACTATTCCTAGGCTTAA.

TABLE I. HLA Phenotype and HCMV Serology of Subjects Studied for gB Specific CTL

Subject	Age (yr)	Sex	HLA class I type	HCMV serostatus
JH	35	F	A1 A11 B8 B55	Positive
NB	23	F	A2 A3 B7 B49	Positive
LU	22	F	A3 A3 B7 B35	Positive
DW	28	F	A3 A11 B16/38 B40	Positive
EW	25	F	A11x Bw60 Bw62	Positive
SW	47	F	A29 A31 B14 B44 B51	Positive
JM	36	F	A1 A2 B8 B44	Positive
JL	23	F	A3 Aw33 B7 B14	Negative

The constructs were transferred and ligated into the vaccinia expression vector pGS20 under the control of the 7.5K early/late promoter. Vaccinia WR-infected CV-1 cells were transfected and gB-expressing viruses selected in the presence of BudR on Rat-2 tk^- cells. Plaques were assayed by Western blotting with the gB-specific monoclonal antibody 27–156 (generous gift of Dr. G. Britt, Alabama) and representative plaques twice plaque-purified and high titre stocks prepared on BHK21 cells infected at M.O.I0.02.

Establishment of Human Fibroblast and B Lymphoblast Lines

Primary human fibroblast lines were established from skin biopsies from HCMV-seropositive and seronegative subjects as previously described [Borysiewicz et al., 1983]. All subjects were HLA-typed by microlymphocytotoxicity tests (National Blood Transfusion Service, Rhydlafa, Wales) [Terasakati et al., 1978]. HLA types are shown in Table I. Fibroblast lines were grown in Eagles' minimum essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) 1% nonessential amino acids (Gibco), 2 mM L-glutamine, 100,000IU/l penicillin and 100 mg/l streptomycin. Fibroblasts used in the experiments described were between passages 4 and 16. Primary HLA-defined B lymphoblastoid cell lines were established as described by Walls and Crawford [1987] and maintained in RPMI-1640 (Gibco) supplemented as described for MEM. All primary cell lines were screened for mycoplasma contamination by enzyme immunoassay (Boehringer Mannhein Biochemica, East Sussex, UK) and stored in liquid nitrogen.

Lymphocyte Culture

PMBC were obtained from HCMV-seropositive and seronegative subjects by ficoll-hypaque gradient centrifugation. They were suspended at 1×10^6 cells/ml in 24-well plates [Greiner, Dursley, UK] in RPMI-1640 with

TABLE II. Summary of HCMV-, IE-1-, and gB-Specific CTL Responses in Polyclonal CTL Cultures

			% Specific lysis of target cells							
			Autologous targets					Allogeneic targets		
Subject	HCMV status	E:T[b]	HCMV[c]	vac.IE-1[d]	vac.gB[d]	Mock[d]	vac.wt[d]	HCMV[c]	vac.IE-1[d]	vac.gB[d]
JH	+	20:1	29 ± 1.4	19 ± 2.3	49 ± 2.5	4 ± 0.5	0 ± 4.1	8 ± 2.5	6 ± 0.5	19 ± 2.6
NB	+	23:1	55 ± 2.4	19 ± 2.1	19 ± 2.3	0 ± 1.2	13 ± 1.8	21 ± 0.9	8 ± 1.1	8 ± 0.2
LU	+	34:1	47 ± 2.2	10 ± 1.1	11 ± 1.3	0 ± 1.9	4 ± 0.8	24 ± 1.8	8 ± 0.6	6 ± 1.7
DW	+	25:1	—[a]	—[a]	51 ± 5.9	—[a]	—[a]	—[a]	—[a]	37 ± 1.0
EW	+	25:1	50 ± 1.6	—[a]	3 ± 0.24	—[a]	—[a]	26 ± 1.5	—[a]	2 ± 0.5
SW	+	12:1	50 ± 4.2	—[a]	0 ± 1.2	—[a]	—[a]	35 ± 2.3	—[a]	1 ± 1.2
JM	+	27:1	44 ± 1.1	12 ± 0.8	15 ± 1.8	4 ± 0.5	3 ± 0.5	8 ± 1.8	6 ± 0.4	18 ± 0.3
JL	−	30:1	34 ± 1.6	37 ± 2.3	38 ± 1.2	23 ± 1.3	40 ± 1.3	31 ± 3.6	34 ± 4.4	36 ± 2.8

[a]Test not done (insufficient effector numbers).
[b]After 14-day stimulation with HCMV-infected (MOI of 10:1, 48 hours) fibroblasts and γ-irradiated autologous PBMC effectors were assayed in standard 5-hour (BCL) or 12-hour (fibroblasts) chromium release cytotoxicity assays at 37°C at effector-to-target ratios (E:T) indicated to determined MHC-restricted CTL killing.
[c]Autologous (autol) and allogeneic (allo) fibroblast target cells were infected with HCMV (MOI of 10:1, 48 hours).
[d]Autologous and allogeneic BCL target cells were infected at MOI of 10:1 for 12 hours with vac.gB, vac.IE-1, and autologous BCL only were infected at MOI of 10:1 for 12 hours with vac.wildtype and mock-infected (mock).

10% human AB pooled serum, glutamine and penicillin/streptamycin as described above.

Autologous fibroblasts infected 48 hours previously with HCMV (MOI of 10:1) together with γ-irradiated (5,000 rads) autologous PBMC were added at 2×10^5 cells/well and 1×10^6 cells/well, respectively. The cultures were incubated for 3 days; when supernatant was removed and fresh media replaced with 10 μ/ml rIL2-conatining medium, medium was replaced every 3 days. After 14 days in vitro, lymphocytes were harvested, washed and used as effector cells in ^{51}Cr-release assays.

Cytotoxicity Assay

The effector cultures were divided equally and cytotoxicity was determined by incubation with 4 to 8 different chromium-loaded target cells at 37°C as described in individual experiments. For each target cell, maximum and spontaneous release values as well as 2-fold dilutions of effector cells were determined. All results were expressed as mean values of triplicates ± 1 S.D.

RESULTS

HCMV-Specific CTL Recognize the 72 kD IE-1 Protein and gB

PBMC from HCMV-seropositive and seronegative subjects were cocultured with 48-hr HCMV-infected autologous fibroblasts and γ-irradiated PBMC feeder cells as described in Materials and Methods. After 14 days, effector cells were assayed against HCMV-infected and uninfected fibroblasts which were both HLA-matched and mismatched to determine the presence of HLA-restricted, HCMV-specific CTL. All 6 HCMV-seropositive subjects tested killed autologous HCMV-infected target cells, whilst in the representative seronegative subject (JL) no specific lysis was detected (Table II).

Previously, a high proportion of HCMV-specific CTL were found to recognise the nonstructural protein IE-1; therefore, cultures were assayed against vac.IE-1 [Borysiewicz, 1988b] and vac.gB [Cranage et al., 1986] infected (12 hour, MOI of 10) autologous and allogeneic BCL. Of 4 HCMV-seropositive subjects with HCMV-specific CTL in PBMC, JH, NB, and JM killed autologous better than HLA-mismatched vac.IE-1-infected target cells. In addition, a greater lysis of autologous vac.gB-infected cells was observed in 4 of 7 subjects (JH, NB, LU, and DW).

PBMC were collected from two pregnant women (JM and JH) on the first trimester, second trimester, at term and 6 weeks postpartum. All samples except those collected postpartum were stored in liquid nitrogen until use. PBMC from each stage during pregnancy were stimulated with HCMV as described previously. Effector cells were assayed against autologous and allogenic HCMV-infected and uninfected fibroblasts (MOI of 10:1, 48 hours) to determine the presence of HLA-restricted, HCMV-specific CTL. Effectors from JM killed HCMV in infected target cells at all stages shown (Table III). Insufficient PBMC from JH were available to assay HCMV-infected targets during first trimester, second trimester or at term; however, HCMV-specific killing was observed postpartum. However, PBMC from JH specifically lysed vac.gB-infected target cells at all stages during pregnancy, suggesting by inference from Table II that HCMV-specific CTL activity was also likely to have been present throughout pregnancy. No specific lysis was detected in the representative HCMV-seronegative subject JL.

Characterisation of Expression of gB Vaccinia Recombinants Encoding gB Truncate

Four recombinant vaccinia viruses (vaccinia WR strain background), containing pGS20 vector sequence, vacgB459, vacgB685, were examined by SDS-PAGE and Western blotting with a pool of polyclonal human anti-HCMV serum and monoclonal antibody 27–156 (provided by Dr. J. Britt) directed against the carboxy part of the gB (gp55) protein.

Vac.gB906 was expressed as both a 140-kd and 55-kd protein, representing the noncleaved gB precursor and the processed carboxyterminal gp55 polypeptide. Vac.gB685 was present as a 33-kd and 110-kd band com-

TABLE III. Summary of HCMV- and gB-Specific CTL Responses in Polyclonal CTL Cultures Throughout Pregnancy

			% Specific lysis of target cells						
			Autologous targets					Allogeneic targets	
Subject	HCMV status	Stage of pregnancy[b]	HCMV[c]	vac.gB[d]	vac.wt (BCL)[d]	$Mock_F$[c]	$Mock_B$[d]	HCMV[c]	vac.gB[d]
JM	+	1st trimester	45 ± 2.1	7 ± 3.0	—[a]	31 ± 0.2	5 ± 0.2	26 ± 3.0	9 ± 0.5
		2nd trimester	32 ± 3.0	6 ± 0.1	—[a]	18 ± 0.1	5 ± 0.3	12 ± 1.5	9 ± 2.0
		Term	41 ± 4.1	4 ± 0.2	—[a]	22 ± 0.6	5 ± 0.5	17 ± 1.1	3 ± 0.3
		Postpartum	37 ± 4.0	11 ± 1.0	—[a]	15 ± 1.6	5 ± 0.3	25 ± 0.6	9 ± 1.0
JH	+	1st trimester	—[a]	37 ± 0.6	12 ± 0.3	—[a]	4 ± 0.1	—[a]	14 ± 0.6
		Term	—[a]	49 ± 0.9	15 ± 0.1	—[a]	11 ± 0.1	—[a]	24 ± 1.0
		Postpartum	30 ± 0.5	46 ± 2.0	3 ± 0.6	—[a]	11 ± 1.2	10 ± 0.5	12 ± 0.7
JL	–	–	24 ± 0.8	15 ± 1.5	6 ± 1.2	13 ± 0.6	7 ± 1.0	22 ± 0.5	11 ± 0.3

[a]Test not done (insufficient effector numbers).
[b]Following 14-day stimulation with HCMV-infected fibroblasts and γ-irradiated autologous PBMC, effector cells collected at different times during pregnancy (1st trimester, 2nd trimester, at term and 6 weeks postpartum) were assayed in standard 5-hour (BCL targets) or 12-hour (fibroblast targets) chromium release cytotoxicity assays at 37°C to determine HLA-restricted CTL killing. All results represent mean of triplicate wells ± 1 S.D. at effector-to-target ratios of 30:1.
[c]Autologous and allogeneic fibroblast target cells were infected with HCMV (MOI of 10:1, 48 hours), autologous fibroblasts were also mock-infected ($mock_F$).
[d]Autologous and allogeneic BCL target cells were infected with vac.gB (MOI of 10:1, 12 hours). Autologous BCL target cells were also infected with vac.wt (MOI of 10:1, 12 hours) and mock-infected with no virus ($mock_B$).

patible with the insertion of a stop codon introduced at amino acid 685 (Fig. 1). This suggests that this truncate gB is processed at the natural processing site (458–459). Vac.gB459 is seen as a broad band approximately 80 kd, probably corresponding to the amino part of gB, eg-gp92-116 kd glycoprotein. This ectodomain (1–459) of gB contains 14 of the 19 putative glycosylation sites. Little secretion of the ectodomain from either Rat-2 or CI-I cells infected with vac.gB906, vac.gB459 was observed.

Finer Specificity of gB Specific CTL Using Vaccinia.gB Truncates

To further investigate the fine specificity of HCMV gB-specific CTL, effector cells were assayed against BCL targets infected with the gB truncates, vac.gB459-21 and vac.gB685. Three of 4 gB responders (JH, NB, and DW Table IV, LU, Table V) responded to either one or both of the truncates in an MHC-restricted manner. JH showed responses to both vac.gB459-21 and vac.gB685. NB recognised vac.gB459-21 only. The third subject, DW, responded exclusively to vac.gB685. JM, who did not recognize gB, showed no response to either of the truncates.

Target Cell Type

Primary human fibroblast cell lines are the only readily available cells fully permissive for HCMV replication in vitro and thus, total HCMV-specific lysis was determined using these target cells. However, in most studies of protein/peptide CTL activity, BCL target cells are used as they are readily maintained and infected with vaccinia recombinants. In order to control for possible differences in CTL epitope processing between these cell lines fibroblasts infected with vac.gB were used as target cells. Cultures from 3 of the 4 subjects with anti-vac.gB CTL activity (Table II) did not kill vac.gB-infected fibroblast target cells (Table V).

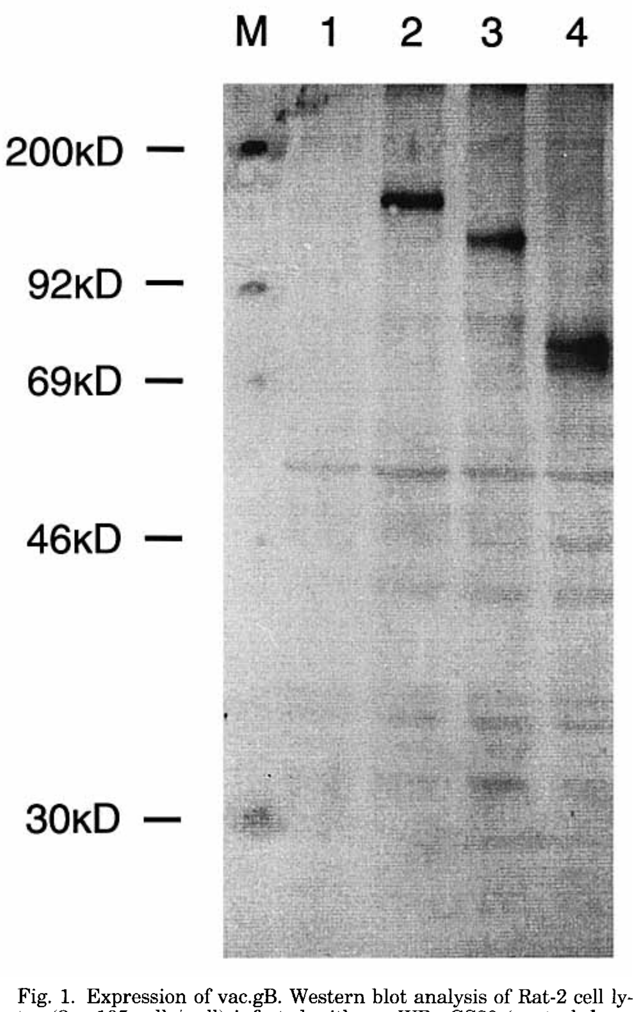

Fig. 1. Expression of vac.gB. Western blot analysis of Rat-2 cell lysates (2 × 105 cells/well) infected with vac.WR-pGS20 (control, **lane 1**), vac.gB906 **(lane 2)** and vac.gB459. Pooled human anti-HCMV serum was used and reaction detected with alkaline phosphatase (Promega Kit, Southhampton, UK).

TABLE IV. Summary of vac.gB-, vac.gB459-21- and vac.gB685-Specific CTL Responses in Polyclonal CTL Cultures

			%Specific lysis of target cells					
			Autologous targets			Allogeneic targets		
Subject	HCMV serostatus	E:T[c]	vac.gB[b]	vac.gB459-21[b]	vac.gB685[b]	vac.gB[b]	vac.gB459-21[b]	vac.gB685[b]
JH	+	12.0:1	29 ± 1.9	46.5 ± 3.0	20.3 ± 1.0	11.6 ± 0.8	9.0 ± 1.9	11.1 ± 1.5
JM	+	10.0:1	17 ± 0.2	5.7 ± 0.6	15.4 ± 2.2*	15 ± 2.8	13.7 ± 1.2	8.3 ± 1.6
NB	+	10.0:1	13.5 ± 1.5	9.1 ± 0.7	9.2 ± 2.3*	7.9 ± 1.1	0.9 ± 1.0	4.1 ± 0.8
DW	+	12.5:1	51.3 ± 6.1	31.4 ± 2.1	46.7 ± 2.0	37.2 ± 4.2	–[a]	–[a]

[a]Test not done (insufficient effector numbers).
[b]Autologous and allogeneic BCL target cells were incubated with vac.gB and the vaccinia gB truncates, vac.gB459-21 and vac.gB685 (MOI of 10:1) at 37°C for 12 hours.
[c]Cytotoxicity was determined in standard 5-hour chromium release assays at effector-to-target ratios (E:T) indicated.
*Student t-test showed no significant difference from allogeneic target cells at $P > 0.05$.

TABLE V. Comparison of HCMV-Specific CTL Killing of vac.gB-Infected BCL and Fibroblast Targets

			% Specific lysis of target cells[b]					
			BCL targets			Fibroblast targets		
Subject	HCMV serostatus	E:T	autol vac.gB[c]	allo vac.gB[c]	autol mock[d]	autol vac.gB[c]	allo vac.gB[c]	autol mock[d]
JH	+	20:1	49 ± 2.5	19 ± 2.6	4 ± 0.9*	22 ± 2.4	19 ± 1.4	26 ± 0.5
NB	+	23:1	19 ± 2.3	08 ± 0.2	0 ± 1.2	32 ± 0.5	23 ± 0.5	0 ± 4.1
LU	+	24:1	11 ± 1.3	06 ± 1.7	0 ± 1.7	09 ± 1.8	11 ± 1.2	12 ± 0.9
JM	+	22:1	15 ± 2.8	17 ± 0.25	4 ± 0.5	18 ± 1.9	14 ± 1.5	13 ± 2.5
DD	+	26:1	–[a]	–[a]	–[a]	05 ± 2.4	05 ± 3.0	–[a]
EW	+	26:1	03 ± 0.24	02 ± 0.5	–[a]	03 ± 2.0	03 ± 1.3	–[a]
JL	–	31:1	34 ± 1.2	31 ± 2.8	24 ± 8.0	31 ± 1.1	30 ± 2.7	25 ± 13.2

[a]Test not done (insufficient effector numbers).
[b]Autologous (autol) and allogeneic (allo) BCL and fibroblast target cells were incubated with vac.gB (MOI of 10:1) at 37°C for 12 hours.
[c]Autologous fibroblast and BCL were mock-infected (mock) and incubated with effector cells at 37°C for 12 hours.
[d]Cytotoxicity determined by standard 5-hour (BCL) or 12-hour (fibroblast) chromium release assays at effector-to-target ratios (E:T) ratios indicated.
*Student t-test showed significant differences from autologous vac.gB-infected targets at $P < 0.05$.

Restimulation and Phenotype of gB-Specific CTL

Since JH showed the greatest gB-specific response, JH gB-specific T cells were further expanded by coculture with vac.gB-infected BCL (MOI of 10, 12 hr), fixed with 2% paraformaldehyde in presence of IL-2 (10 μ/ml) until day 35. The phenotype of cells following 28 days in culture was 99%CD3+, 96%CD4+ and 11%CD8+ by single colour flow cytometry (data not shown). When these cells were assayed in chromium release assays, the effector cells lysed vac.gB-infected BCL targets but not autologous HCMV-infected fibroblasts (Fig. 2).

MHC Class I Restriction

To confirm the MHC class II restriction of JH gB-specific CTL, HCMV-specific bulk cultures were assayed against MHC class I one allele-matched BCL target cells infected with either vac.gB459 or vac.gB685. There was no specific cytotoxicity against any of the targets used apart from autologous vac.gB459-infected BCL (Fig. 2) implying the predominance of the CD4 phenotype, and failure to kill HCMV-infected fibroblasts MHC class II restricted cytotoxicity.

DISCUSSION

HCMV gB is a candidate subunit vaccine against HCMV, based on its ability to generate a neutralising antibody response. In this study, cytotoxic T cell responses against HCMV gB were examined to determine whether during natural persistant infection, as pertains in 60–90% of the population, this immune response is efficiently sustained and demonstrable in vitro.

It was found previously that using a vaccinia recombinant encoding the HCMV glycoprotein B, [Borysiewicz et al., 1988b] that HCMV-specific CTL from subjects persistently infected with HCMV recognised gB. Furthermore, these CTLp were present in PBMC of normal HCMV-seropositive individuals, but at a very low level. Our results are consistent with a low proportion of gB-specific CTL observed as only 4 of 7 HCMV-specific CTL cultures recognised vac.gB in a MHC restricted manner. An HCMV IE-1-specific CTL cytotoxic response was detected in three of four individuals tested. As reported previously [Borysiewicz, et al., 1988a] this IE-1 specific lysis was observed after secondary in vitro stimulation with HCMV-infected fibroblasts. Although IE-1 may block its own presentation by MHC class I [Gilbert et al., 1993], this block cannot be absolute, as IE-1-specific CTL can still be generated with HCMV infection.

HCMV-specific HLA-restricted cytotoxic lymphocyte activity was demonstrated during each trimester of pregnancy in JM and at postpartum in JH. Vac.gB-specific HLA-restricted cytotoxic activity was detected in subject JH both early and late in pregnancy. As gB-specific CTL

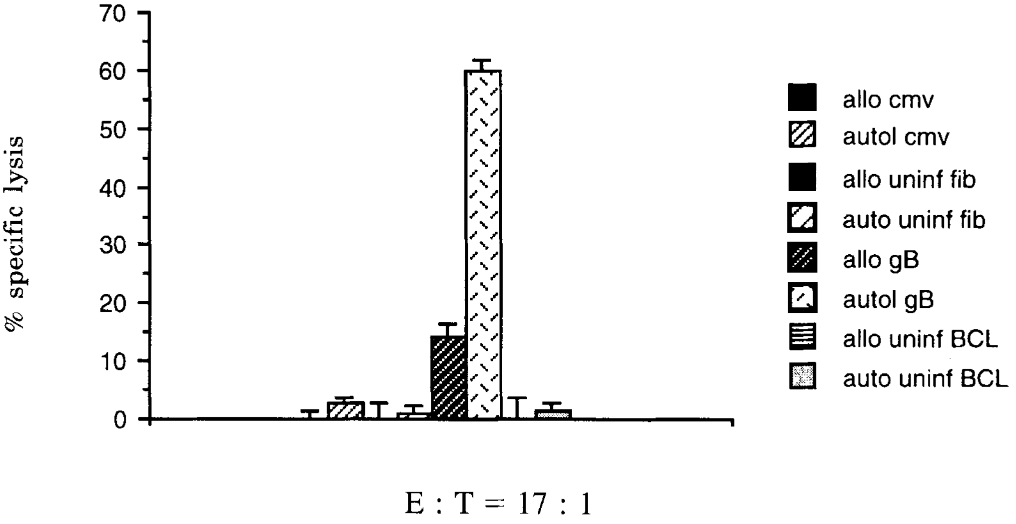

Fig. 2. gB-specific CTL response of subject JH. CTL response of JH on day 28 following stimulation of 14-day bulk culture with autologous vac.gB-infected BCL stimulator cells and allogenic PBMC feeder cells. Specific lysis of target cells was determined by ^{51}Cr release assay. Results represent the mean values of triplicates ±1 SD.

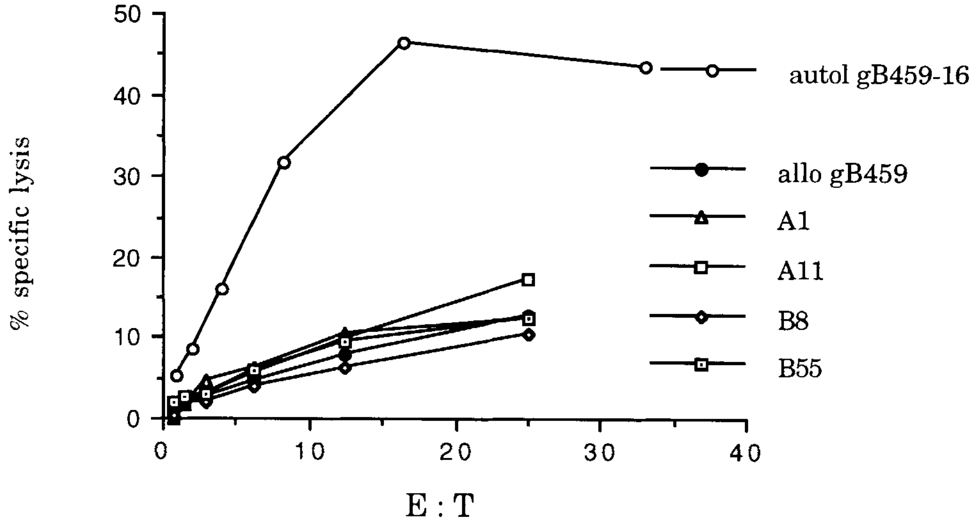

Fig. 3. gB-specific CTL response to vac.gb459-21- and vac.gB685-infected MHC class I; one allele matched BCL targets in subject JH. Specific lysis of JH 14-day effector cells following stimulation of PBMC with autologous HCMV-infected (MOI of 10:1, 48 hr) fibroblasts, determined in standard 5-hour Cr release cytotoxicity assay. Lysis of autologous (open circle), allogenic (closed circle) and one-allele MHC class I-matched (A1, open triangle; A11, open square; B8, open diamond; B55, semi-closed square) vac.gB459-21-infected BCL targets (MOI of 10:1, 12 hr).

activity was only observed in the presence of HCMV-specific CTL (Table I), it is probable that such activity was also present earlier in pregnancy. Therefore, in these two subjects, HCMV CTL responses were preserved throughout pregnancy, suggesting that if induced earlier, CTLp can be detected and may play a role in controlling spread of virus to the fetus.

We used vaccinia truncates to delineate further the fine specificity of gB-specific CTL. Two of the four cultures assayed against the gB truncates showed weak cytotoxic activity towards vac.gB685-infected targets cells, suggesting there may be a gB-specific CTL epitope on the gp55 or C terminal region of gB. This result may be consistent with observations reported by Liu et al. [1991] that the gB-specific CTL response includes CTL-recognising epitopes on the N-terminal 513 amino acids of gB. Therefore, it is possible that the gB-specific CTL epitope is situated between amino acids 459 and 685. However, allogeneic and partially matched target cells were not used in those CTL assays described by Liu et al. [1991]; therefore, this MHC restricting element is unknown (see below).

In contrast, Utz et al. [1992] reported an HLA-A2-restricted CTL epitope at amino acid residues 619–628 in the C terminal region of the gB complex. We did not find this response in JH (HLA A2 +ve), as no specific cytolytic activity against vac.gB685 was observed, although the vac.gB459–21 N-terminal truncate was lysed. It is likely that CTL epitopes, both MHC class I- and class II-restricted, are located throughout the structure of gB, and small fragments of gB may fail to induce humoral and cellular immunity in all subjects in an outbred population.

When HCMV-specific CTL from all four subjects who lysed vac.gB-infected BCL targets were assayed against the vac.gB-infected fibroblasts, no lysis was observed in three of four. As MHC class II is not expressed on fibroblasts, even following HCMV infection, CTL in these three subjects could be predominantly restricted by MHC class II rather than class I. This was confirmed to be the case for subject JH. This does not imply dominance but suggests that in future studies, predominance of CD4 must be considered.

Recently, Curtsinger et al. [1993] demonstrated that the magnitude of the in vitro T cell proliferative response to gB is determined by the HLA-DR haplotype of the donor. By analysis of polyclonal gB-specific T cell lines from HLA-DR heterozygous subjects, they confirmed that the two alleles chosen for study (DR4Dw4 and DR3Dw3) were associated with low proliferative response to gB and that DR4Dw10 and DR7Dw7 were high gB responder alleles. It is interesting to note that in previous work showing the precursor frequency of gB-specific CTL [Alp et al., 1991], the subject was HLA-DR4 and in this study, two of four gB responders are HLA-DR4 (the DR haplotype of the remaining two is unknown). Thus, a proportion of the DR-restricted responding cells described by Curtsinger et al. [1993] could by cytotoxic.

This study suggests that in normal asymptomatic subjects the MHC class II-restricted response against gB may predominate. MHC class I-restricted responses against gB can be observed. Borysiewicz et al. [1988b] observed lysis of vac.gB-infected BCL targets by HCMV-specific CTL as well as gB-specific CTL (PBMC stimulated with vac.gB-infected fibroblasts for 14 days). As their experiments were performed in limiting dilution against BCL targets, this precise MHC restriction was not defined. Gilbert et al. [1993] reported a gB-specific CTL cytolytic response when HCMV-specific CTL were assayed against vac.gB-infected fibroblast targets, strongly suggestive of MHC class I restriction, although the restricting element was not fully defined. This study suggests that care must be taken especially when BCL target cells are used, to define the restricting element accurately. Whilst it is clear that MHC class I-restricted CTL against gB are present [Borysiewicz et al., 1988b; Gilbert et al., 1993; Utz et al., 1992], these may not be the numerically dominant CTL population against gB.

Cytotoxic lymphocyte activity appears crucial in the protection of the fetus from intrauterine infection with HCMV [Stern et al., 1986], and it is widely held that potential vaccine candidates should be capable of inducing this response as well as neutralising antibody to extracellular virions. There are some concerns over using live virus vaccines in view of the latent nature of CMV infection which might at some point reactivate to produce clinical disease at times of immunosuppression including pregnancy [Plotkin et al., 1990]. Work into elucidating epitopes capable of producing CTL responses for subunit vaccines is continuing. A combination subunit vaccine encoding both a viral glycoprotein for neutralising antibody and IE-1 or pp65 to generate virus-specific CTL should be considered. This study indicates that if HCMV-specific CTLp are present, their demonstrable activity is maintained during pregnancy; a vaccine-induced response will be retained during pregnancy many years after the primary contact.

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